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Biochimica et Biophysica Acta 1544 (2001) 255–266

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Towards molecularly imprinted polymers selective to peptides and proteins. The epitope approach

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Received 8 February 2000; received in revised form 5 September 2000; accepted 19 September 2000

Abstract

In this paper, we describe the epitope approach to molecular imprinting. The applicability of molecular imprinting, a method that allows the preparation of biomimetic compounds (artificial receptors and antibodies), is extended by this approach. Our approach makes it possible to obtain imprinted polymers selective to peptides and proteins whereas, to date, molecular imprinting has been used primarily for the preparation of polymers that selectively bind to relatively low molecular weight substances. The epitope approach is based on using (as a template) a short peptide that represents only part of a larger peptide or protein (as an epitope represents an antigen), which in turn can be recognized by the synthesized polymer. It is demonstrated that although other parts of peptides can influence the process of molecular recognition, the polymers imprinted with a short peptide efficiently recognize both the template and larger peptides (for example, oxytocin) that possess the same C-terminal part of the structure.

Keywords: Molecular recognition; Imprinted polymer; Epitope approach; Oxytocin; Circular dichroism; Peptide conformation

1. Introduction

Molecular imprinting is a method for preparing polymers of predetermined selectivity for the separation and analysis of a vast variety of biologically active or harmful substances [1–4]. The technique involves the formation of complexes between a print molecule (template) and a functional monomer based on relatively weak, noncovalent interactions (hydrogen bonding, ionic, hydrophobic, etc.). These complexes appear spontaneously in the liquid phase and are then fixed sterically by polymerization with a high degree of cross-linking. After extracting the print molecules from the synthesized polymer, free recognition sites, which are able to recognize the template during subsequent rebinding procedures, remain in the polymer matrix. Chemically and mechanically stable molecularly imprinted polymers (MIPs) able to recognize specific substances may successfully serve as substitutes for antibodies, enzymes or other native biological structures in fundamental investigations of molecular recognition and may have numerous other applications in biotechnology, medicine, environmental control, etc.

Usually, only relatively low molecular weight compounds (sugars, steroids, amino acid derivatives, certain drugs and pesticides) are used as templates. To date, at least two effects have been reported to hinder

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0167-4838/01/$ – see front matter
PII: S0167-4838(00)00226-0

the synthesis of MIPs selective to macromolecules such as proteins: steric and thermodynamic. The steric effect is based on the notion that bulky protein templates cannot slip in and out of a polymer network [5]. Therefore, attempts to synthesize protein selective MIPs have been mainly associated with the so-called surface imprinting procedure. Unfortunately, this procedure, using metal (Cu^{+2}) chelating monomers, can be applied only to proteins containing exposed histidine residues [6,7]. However, the physical structure of macroporous MIPs provides another means of overcoming this problem. It is well known that the diameters of globular proteins having one polypeptide chain and proteins possessing a more complex structure (for example, hemoglobin) do not usually exceed 5–10 nm. Macroporous MIPs have been shown to possess a significant internal surface area (250–500 $m^2\ g^{-1}$) and a broad distribution of large pores (> 50 nm) [3], which ensures that not only solvents and low molecular weight substances, but also peptides and proteins have access to an essential fraction of the polymer mass. According to Wulff [1], the specific microcavities formed by the imprinting process (about 0.5–1.5 nm in diameter) are located on the surfaces of the above-mentioned large pores.

Thermodynamic considerations [8] indicate that the use of non-rigid templates, such as polypeptides or proteins, yield less well-defined recognition sites in MIPs. Indeed, attempts to involve the protein itself in the polymerization process by entrapping it in polysiloxane or in polyacrylamide polymers [9–12] have been characterized by low specificity and unsatisfactory reproducibility. On the other hand, Mosbach et al. [13–15] have demonstrated the efficient recognition of short oligopeptides (3–5 amino acid residues) by MIPs imprinted with these oligopeptides.

These considerations suggest a general method for the preparation of MIPs specific to polypeptides and proteins: if a short peptide representing only a small exposed fragment of a protein structure is used as a template, then the resultant macroporous MIP recognizing the imprinted peptide will also be able to recognize the whole protein molecule (Fig. 1). We have termed this method the 'epitope approach', from the fact that a similar means is employed by nature; in recognizing an antigen, an antibody interacts with only a small part of it, the epitope (the antigenic site of macromolecule).

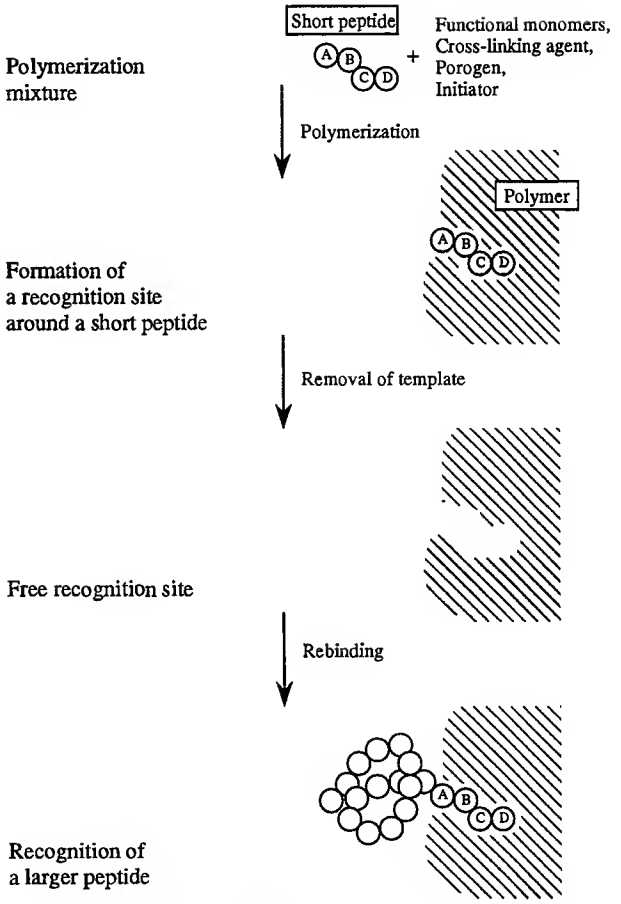

Fig. 1. Schematic representation of the epitope approach.

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 2,2′-azobis(2,4-dimethylvaleronitrile) were obtained from Wako (Osaka, Japan). Tyr-Pro-Leu-Gly amide (YPLG), oxytocin, tocinoic acid, *N*-benzyloxycarbonyl-Pro-Leu-Gly amide (Z-PLG) and Gly-Leu-Tyr (GLY) were obtained from Sigma (St. Louis, MO, USA), mesotocin or (Ile^8)-oxytocin from Bachem (Bubendorf, Switzerland), and deamino oxytocin (DeAm-Oxy) from Biogenesis (Poole, UK). [$Asu^{1,6}$]-oxytocin (Asu: L-α-aminosuberic acid), acetyl-L-tyrosine amide (Ac-

Tyr-NH_2), acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), leucine-enkephalin (YGGPL), β-casomorphin-5 (YPFPG) and Pro-Leu-Gly amide (PLG) were purchased from the Peptide Institute (Osaka, Japan). The acetonitrile used was of high performance liquid chromatography (HPLC) grade. Water was of Milli Q grade.

2.2. Polymerization

A bulk radical polymerization was carried out at 40°C for 16 h using 2,2′-azobis(2,4-dimethylvaleronitrile) as the initiator. The polymers obtained were ground in a mortar and sieved to collect the 20–45 μm fraction. The polymers were washed several times with acetonitrile and water containing 5 vol% acetic acid until the template could no longer be detected in the supernatant. Control (non-imprinted) polymers were synthesized under the same conditions, though in the absence of template.

2.3. Chromatographic evaluation

Chromatographic analyses were performed using a Tosoh 8010 HPLC system (Tosoh, Tokyo, Japan), which included a system controller and UV detector. Polymers were slurry-packed into 100×4.6 mm stainless steel columns. Samples (20 μl, 0.05 mM) were analyzed at a flow rate of 1.5 or 3 ml min^{-1} and monitored with the UV detector set at 225 or 275 nm (using acetone or $NaNO_3$ as a void marker). The capacity factor (k') was calculated as $(t-t_0)/t_0$, where t is the retention time of the solute and t_0 is the retention time of the void marker; the separation factor of a given non-template analyte was defined as $\alpha = k'_t/k'_x$, where k'_t and k'_x are the capacity factors of template and other compound, respectively [16].

2.4. Measurements of circular dichroism (CD)

CD spectra were obtained at room temperature (~25°C) on a JASCO model J-720 spectropolarimeter using a cylindrical fused quartz cell with an optical path length of 1.0 cm. Solutions were prepared by weighing lyophilized substances; concentrations were about 0.03 mM. The solvent baseline was subtracted, and spectra were normalized in units of molar ellipticity [θ] (deg cm^2 $dmol^{-1}$).

3. Results and discussion

To explore the epitope approach, we chose the neurohypophyseal hormone, oxytocin. It plays the role of instigator of the birth process in mammals, enabling a pregnant female to start uterine contractions during labor, to begin lactating, to bond with her newborn and, in general, to behave maternally. Oxytocin is believed to be responsible for initiating sexual receptivity in both males and females and for the bonding behavior between them (called 'pair-bonding'). Recently, it was reported that an imbalance in the production of oxytocin and opioid peptides, with a prevalence of opioid peptides, might underlie a condition of sexual impotence [17].

Oxytocin is a nonapeptide (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2). The formation of a disulfide bond between the Cys residues at positions 1 and 6 results in a peptide consisting of a 6-amino acid cyclic portion and a 3-amino acid C-terminus. Despite considerable progress in elucidating the structure of the oxytocin receptor, many aspects of the interaction between oxytocin and its receptor remain poorly understood [18]. However, numerous enzymatic and immunological studies indicate a key importance of the 3-amino acid acyclic tail in mediating intermolecular interactions. For example, the biological activity of oxytocin (but not vasopressin) is destroyed by chymotrypsin, which acts at the leucine–glycine amide bond. It has also been shown that the antigenic site (epitope) of oxytocin is located at its C-terminus, since oxytocin antibodies cannot inhibit structurally similar hormones with a substitution at position 8 [19]. It should be also noted that conversion of the C-terminal glycinamide group into a dimethylamide leads to a drastic reduction in both affinity to the receptor and the hormone's intrinsic activity [20].

These features of oxytocin make it suitable for our investigation. It should be noted that epitopes on an antigen can usually be described as surface domains composed of three to six amino acid residues with a surface area buried in the process of antigen–antibody interactions in the order of 650–900 $Å^2$ [21,22]. The area buried during the folding of a protein or in an antigen–antibody interaction is directly related to the hydrophobic energy that helps compensate for the loss of conformational entropy. It is

known [23,24] that for an unfolded polypeptide chain, the total accessible surface area A_t in an extended conformation is directly proportional to its molecular weight:

$$A_t = 1.45M_w$$

Therefore, this value for the tripeptide tail region of oxytocin is about 450 Å^2, somewhat smaller than is usually needed. Moreover, PLG weakly absorbs UV light, making the control of polymer processing and further binding studies somewhat difficult. To overcome this problem, in our experiment we used as a template the tetrapeptide, YPLG. Its total accessible surface area is about 650 Å^2, and the energy of hydrophobic interactions in combination with the energy of hydrogen bonding with functional monomers should be enough for efficient retention (recognition) of the peptides by an imprinted polymer. In addition, YPLG contains a Tyr residue, which can facilitate its UV and fluorescence detection. It was also expected, that the bulkiness of the Tyr residue would further the formation of the recognition sites, which should recognize not only the template but also other peptides, including oxytocin, with a PLG C-terminal tripeptide.

In view of the fact that a highly cross-linked macroporous copolymer of MAA as a functional monomer and EGDMA as a cross-linking agent has proved to be a very efficient matrix in the molecular recognition process by MIPs [14,25–27], we used these compounds in our investigation.

The usual quantity of relatively low molecular weight template required for MIP preparation is about 1 mmol; the optimal ratio of the template to functional monomer is reported to be from 1:4 to 1:18 [14,26,27] or about four molecules of functional monomer per functional group of a template. To synthesize an anti-[Leu^5]enkephalin MIP, only 0.1 mmol of the template was used [14]. We performed the polymerization with a smaller quantity (22 μmol) of YPLG. YPLG possesses about 10 potential sites for hydrogen bonding. Therefore, we employed template to functional monomer ratios of 1:10, 1:20 and 1:40. For all the synthesized polymers, 6.6 mmol EGDMA was used, and as a consequence, the functional monomer to cross-linking agent ratios ranged from 1:30 to 1:7.5. Although the success of molecular imprinting is generally associated with the use of inert, nonpolar porogens such as chloroform or toluene [1,3,28], it is necessary to consider not only the polarity of the solvent but also the likelihood of it interacting with solute species, e.g. through hydrogen bonding [4,29]. Therefore, for this study, acetonitrile was expected to be a more suitable porogen than, for example, chloroform. To ensure complete solubilization of the YPLG, we added a small quantity of water (about 3 vol%) to the final polymerization mixture, which contained equal volumes of acetonitrile and monomers (MAA+EGDMA).

Elemental analysis of the resultant polymers was in good agreement with the composition described above. For example, for MIP 1:10 we calculate 60.43% C and 7.10% H and found 59.15% C and 6.91% H. The analysis of nitrogen content indicated almost complete removal of the template from the imprinted polymers.

Following polymerization and processing, the properties of the synthesized polymers were evaluated by HPLC. At first, this was done in the aqueous-poor mobile phase, 10 vol% H_2O/90 vol% acetonitrile, modified by 1 mM acetic acid (without adjustment of pH). The results of the chromato-

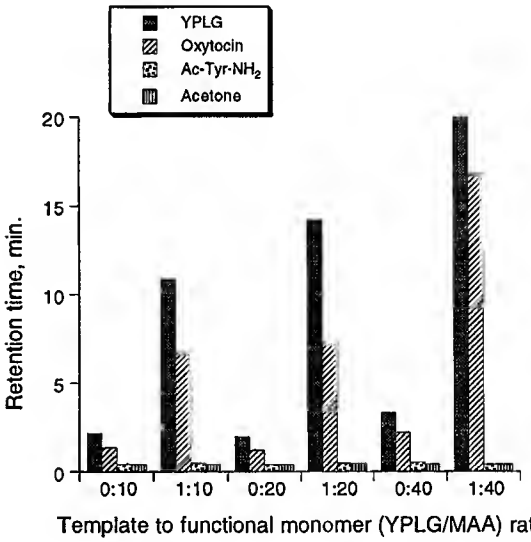

Fig. 2. Chromatographic evaluation of the synthesized polymers. 1:10, 1:20 and 1:40 indicate imprinted polymers having these ratios of template to functional monomer (YPLG/MAA). 0:10, 0:20 and 0:40 indicate the corresponding control (non-imprinted) polymers. The flow rate was 3 ml min^{-1}. The mobile phase consisted of 1 mM acetic acid/10 vol% H_2O/90 vol% acetonitrile. UV detection occurred at 275 nm.

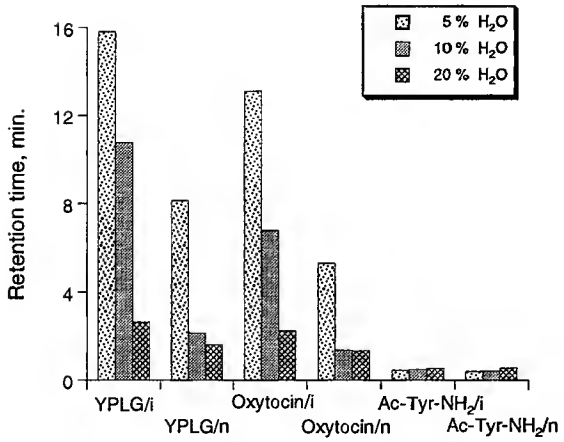

Fig. 3. Influence of the water content of the mobile phase on the degree of interaction between the analytes and MIP 1:10 (analyte/i) or the corresponding control polymer (analyte/n). The flow rate was 3 ml min^{-1}. The mobile phase consisted of the indicated concentration of water in acetonitrile containing 1 mM acetic acid (final concentration). UV detection occurred at 275 nm.

graphic evaluation of the synthesized polymers (Fig. 2) show successful imprinting with YPLG, since the MIPs retained YPLG much strongly than did the non-imprinted polymers. It is of primary importance that MIPs imprinted with the tetrapeptide (YPLG) also recognized oxytocin, a larger molecule bearing the same 3-amino acid C-terminus as that of the template used. In contrast to YPLG and oxytocin, Ac-Tyr-NH_2 essentially did not interact with any of the synthesized polymers.

In view of the facts that an increase in MAA content led to a longer peptide retention and that non-specific interactions with the non-imprinted polymer 0:40 (template to MAA ratio) were higher than those of the two other control polymers, we conclude that the optimal ratio of the template to functional monomer lies between 1:10 and 1:20. This observation suggests that of all the potential hydrogen bonding sites of YPLG, only some participate simultaneously in intermolecular interactions with MAA to form specific recognition sites within MIPs.

Increasing the water content of the mobile phase, as expected, weakened the hydrogen bonding between polymer and analyte. This resulted in a dramatic change in the retention of the peptides by both the imprinted and non-imprinted polymers, whereas the level of interaction between both polymers and Ac-Tyr-NH_2 remained the same (Fig. 3). Ten percent water content in the aqueous-poor mobile phase was used in further experiments because it was a compromise between lower water concentrations, which assured a higher level of hydrogen bonding but limited solubility of the tested peptides, and higher water concentrations, which assured good peptide solubility but restricted hydrogen bonding.

In the next stage of this investigation, we used a set of template- and oxytocin-related peptides to reveal selectivity of the MIP (Fig. 4).

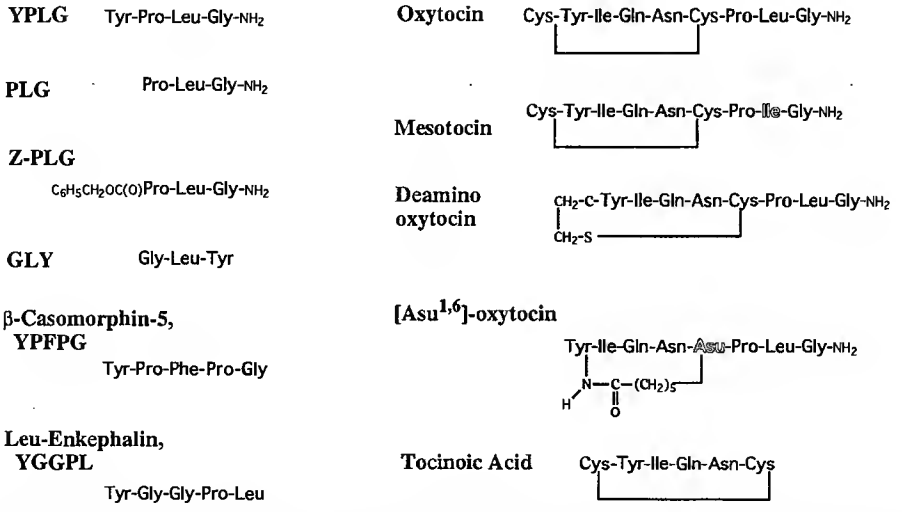

Fig. 4. Amino acid sequences of the tested peptides. Amino acid substitutions (compared with oxytocin) are designated in outline. [$Asu^{1,6}$]-oxytocin: cyclic form between Asu ω-carboxyl group and Tyr α-amino group. Asu: L-α-aminosuberic acid.

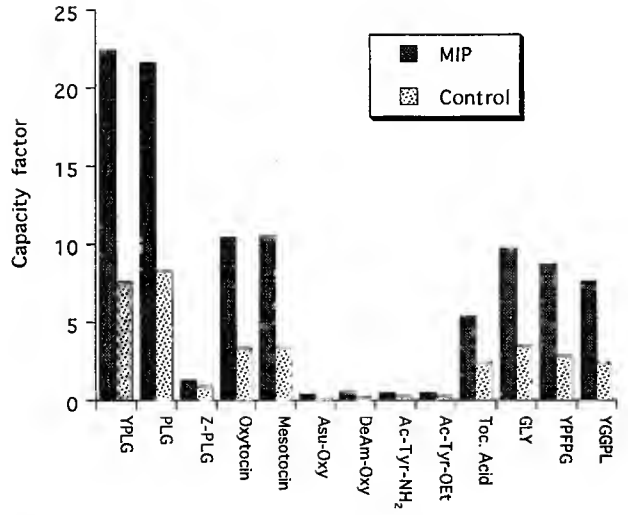

Fig. 5. Chromatography of peptides structurally related to the template or oxytocin by MIP 1:10 and by the corresponding control polymer in the aqueous-poor mobile phase (10 vol% H_2O/90 vol% acetonitrile, modified by 1 mM acetic acid, pH 5.0). The flow rate was 1.5 ml min^{-1}. UV detection occurred at 225 nm.

In the aqueous-poor mobile phase (10% H_2O/90% acetonitrile, modified by 1 mM acetic acid, pH 5.0) the strong retention of the template YPLG, as well as that of PLG, oxytocin and mesotocin, by the MIP corresponds completely to the proposed epitope approach of molecular imprinting. The same holds for the very weak retention of the tyrosine derivatives, Ac-Tyr-NH_2 and Ac-Tyr-OEt (Fig. 5). However, we did not expect the very weak retention of the peptides possessing the C-terminal PLG sequence (deamino oxytocin and [Asu[1,6]]-oxytocin) or the rather strong retention of peptides whose structures do not contain this sequence but do contain the N-terminal amino group (tocinoic acid, GLY, YPFPG and YGGPL).

To get an insight into the nature of the interactions, we investigated the influence of the composition of the mobile phase. It appears that a chromatographic mobile phase containing 2–5% acetic acid ensures the protonation of MAA residues within polymers and thereby reduces the role of ionic interactions [14,30]. Here, by using a much lower concentration of acetic acid (from 1 to 10 mM or from 0.006 to 0.06 vol%), we tried to approach the initial polymerization conditions and, consequently, the initial interactions (Fig. 6). The lower the concentration of acetic acid, the better the recognition of the peptides by the MIP is. Along with increased retention, we also observed a selectivity advantage of the template (YPLG) over the other tested peptides. The only exception was the retention of PLG, which was very similar to that of the template YPLG. The values of the separation factor, α, gradually increased, for example, from 1.48 to 1.85 for oxytocin and from 1.80 to 2.87 for tocinoic acid.

Changing the pH of the aqueous-poor mobile phase from pH 4 to 7 at all tested concentrations of acetic acid led to a progressive decrease of the retention of all analytes; however, the change in selectivity tended to be dependent on the analyte (Table 1). For example, PLG could not be separated from the template YPLG (with the only exception at pH 7 and 2.5 mM acetic acid: under these conditions, PLG was retained twice as strongly as the template). The highest value of the separation factor for tocinoic acid was achieved at pH 7 and 5 mM acetic acid; for oxytocin and the tripeptide GLY, the highest values were at pH 6 and 2.5 mM acetic acid. The differences in dependence on pH can be explained, at least partially, by the different pK_a values of the N-terminal amino groups belonging to proline, tyrosine, glycine and cysteine.

Our investigations of the MIP–peptide interactions in an aqueous-rich mobile phase [31] also demon-

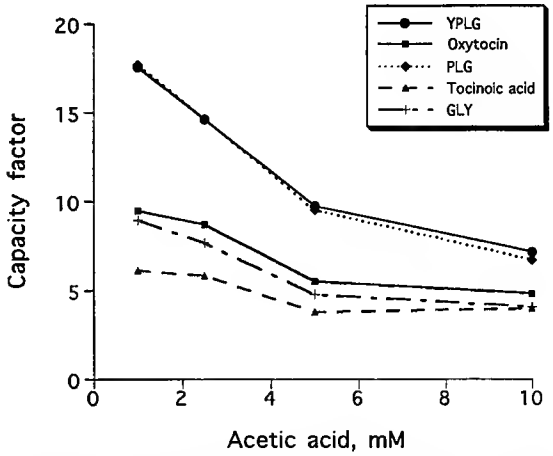

Fig. 6. Influence of the acetic acid content of the aqueous-poor mobile phase on the degree of interaction between the analytes and the MIP 1:10. The flow rate was 1.5 ml min^{-1}. The mobile phase consisted of the indicated concentration of acetic acid in 10 vol% H_2O/90 vol% acetonitrile, pH 5. UV detection occurred at 225 nm.

strated the influence of ionic strength and a clear dependence on the pK_a values of the N-terminal amino acid. However, the pH values corresponding to the strongest retentions of the tested peptides were observed to be higher than 5.5. This difference can be associated with the fact that the pH value for a specific acid–base buffer varies with the solvent composition. For example, it has been shown that the pH values of a 0.01 M acetate buffer in water and in the mixture of 40% water and 60% acetonitrile differ by almost two pH units [32]. Our titration curves (Fig. 7) show that in the mixture of 10% water and 90% acetonitrile, measured values of pH 4 and 5 corresponded not to an approximately half-ionized state of acetic acid but to an almost completely protonated one. The same is probably true for the carboxy groups of MAA residues within the synthesized polymers. It means that ionic interactions do not play a significant role in retention of the peptides by the MIP in the aqueous-poor mobile phase at pH 4, even at low concentrations (1–10 mM) of acetic acid. Moreover, essential deprotonation of carboxy groups at pH 6 and 7 did not lead to the strengthening of the peptide retention.

It should be noted that the increase of pH weakened retention of YPLG much more slowly than that of peptides not possessing the C-terminal PLG sequence. Oxytocin, whose structure includes both the specific and nonspecific fragments, is intermediate in behavior. This could mean that the correct localization of the N-terminal amino group and the C-terminal PLG sequence (such as takes place in the structure of the template YPLG) is very important for peptide recognition by the MIP in the aqueous-poor mobile phase.

Unlike the case in the aqueous-poor mobile phase,

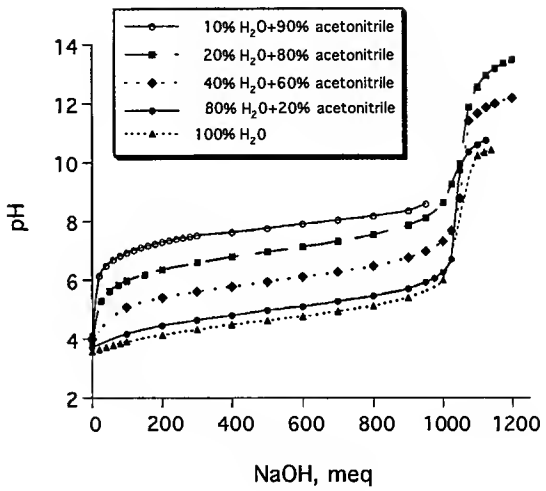

Fig. 7. Titration of 5 mM acetic acid in different mixtures of water and acetonitrile.

Table 1
Dependence of HPLC data for the MIP on concentration of acetic acid and pH[a]

Acetic acid (mM)	Analytes	pH 4		pH 5		pH 6		pH 7	
		k'_i	α	k'_i	α	k'_i	α	k'_i	α
2.5	YPLG	19.87		14.63		7.35		1.86	
	oxytocin	16.98	1.17	8.70	1.68	2.02	3.64	0.57	3.26
	PLG	18.35	1.08	14.65	1.00	9.00	0.82	3.83	0.49
	tocinoic acid	12.13	1.64	5.84	2.51	1.81	4.06	0.40	4.65
	GLY	14.87	1.34	7.68	1.90	1.98	3.71	0.74	2.51
5	YPLG	12.13		9.73		4.56		1.80	
	oxytocin	11.78	1.03	5.51	1.77	1.40	3.26	0.80	2.25
	PLG	12.05	1.01	9.51	1.02	5.02	0.91	2.38	0.76
	tocinoic acid	9.63	1.26	3.78	2.57	1.28	3.56	0.37	4.86
	GLY	10.72	1.13	4.76	2.04	1.33	3.43	0.63	2.86
10	YPLG	9.68		7.15		3.35		1.25	
	oxytocin	9.25	1.05	4.82	1.48	1.19	2.82	0.60	2.08
	PLG	8.68	1.12	6.71	1.07	3.51	0.95	1.44	0.87
	tocinoic acid	8.15	1.19	3.98	1.80	1.25	2.68	0.29	4.31
	GLY	8.15	1.19	4.05	1.77	1.14	2.94	0.48	2.60

[a]The mobile phase consisted of 10% H_2O/90% acetonitrile. Flow rate was 1.5 ml min^{-1}.

the study of the MIP in the aqueous-rich mobile phase containing a citrate–phosphate buffer [31] showed that oxytocin analogs, whose structures contain the C-terminal PLG sequence but not the N-terminal amino group, could also be specifically recognized by the MIP. Here, we demonstrate a similar result using an aqueous-rich mobile phase containing an acetate buffer (Fig. 8). It should be noted that under this condition, the peptides without the C-terminal PLG sequence (YGGPL, YPFPG, GLY and tocinoic acid) are retained by the MIP much more weakly than deamino oxytocin and [Asu^{1,6}]-oxytocin. Another difference from the results obtained in the aqueous-poor mobile phase is the striking retention of Ac-Tyr-OEt by the MIP; its k'_i value increased by more than seven times, but it was retained by the control polymer at the close level. Consequently, this phenomenon can be easily explained by nonspecific hydrophobic interactions with the polymer matrix. In fact, Z-PLG is an example of the strengthening of the interaction of the specific tripeptide segment in the aqueous-rich mobile phase by hydrophobic substitution of the N-terminal amino group (compare retentions of Z-PLG and PLG).

To understand the reason for the difference in the recognition of deamino oxytocin and [Asu^{1,6}]-oxytocin by the MIP, depending on the composition of the

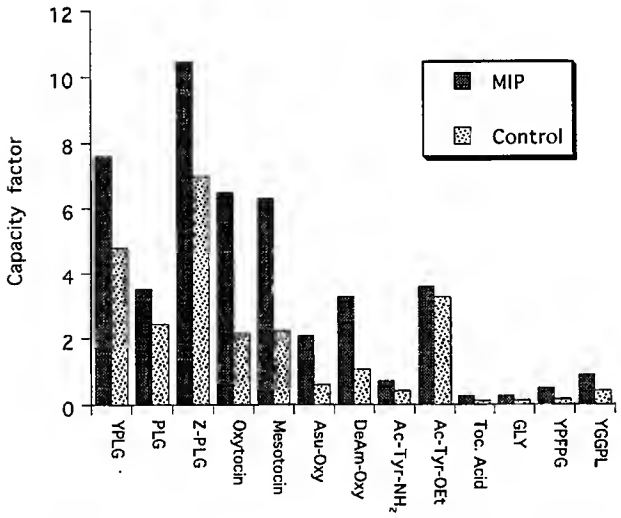

Fig. 8. Chromatography of peptides structurally related to the template or oxytocin by MIP 1:10 and by corresponding control polymer in the aqueous-rich mobile phase (80 vol% H_2O/20 vol% acetonitrile, modified by 2 mM acetic acid, pH 6.5). The flow rate was 1.5 ml min^{-1}. UV detection occurred at 225 nm.

mobile phase, we should also take into consideration the flexibility of conformation of the oxytocin and its analogs. The conformation of oxytocin and its analogs has been the subject of intensive investigation using theoretical [33–35], X-ray diffraction [36,37], CD [38–40], Raman [41,42] and nuclear magnetic resonance studies [34,43–45]. It was shown that the hormone can adopt conformations that are stabilized by intramolecular hydrogen bonds in the hexapeptide ring moiety and the tripeptide tail region [46]. The crystal structure (deamino oxytocin) is characterized by a type II β turn within the ring stabilized by two *trans*-annular hydrogen bonds between Tyr^2 and Asn^5 and a weak hydrogen bond between the Cys^6 CO and the Gly^9 peptide NH, which holds the tripeptide tail in the type I β turn [37].

The conformation of oxytocin in solution depends on the nature of the solvent. β turns stabilized by hydrogen bonds are maintained in organic solvents (dimethyl sulfoxide and trifluoroethanol) [34,44,46]; acetonitrile can also promote the formation of ordered structures [47]. However, unlike these solvents, in an aqueous solution, oxytocin molecules are extremely flexible and do not appear to participate in intramolecular hydrogen bonding [33,41,43,48]. Moreover, it has been shown that there is considerable intramolecular motion in the tail region of oxytocin with respect to the remainder of the molecule [45,49].

CD is a widely used spectroscopic technique valuable for studying secondary structures of oxytocin and its analogs in solution. Such molecules are not sufficiently large that their CD spectra can be interpreted in terms of regular structures such as helical conformations. Usually, their CD spectra include a strong negative band near 200 nm (due to an amide π–π^* transition) and a weak positive band near 220 nm that corresponds to unordered polypeptide structures [50]. On the other hand, electronic transitions of peptide and disulfide bonds, as well as aromatic chromophores of tyrosine, can produce a number of distinct bands in the CD curves. In spite of some differences in interpretation by different authors, it is possible to make assignments for the extremely wide positive band in the 215–230 nm range, the positive band at about 250 nm, and the negative band near 280 nm. The first band results from the superposition of two bands: the longer wavelength

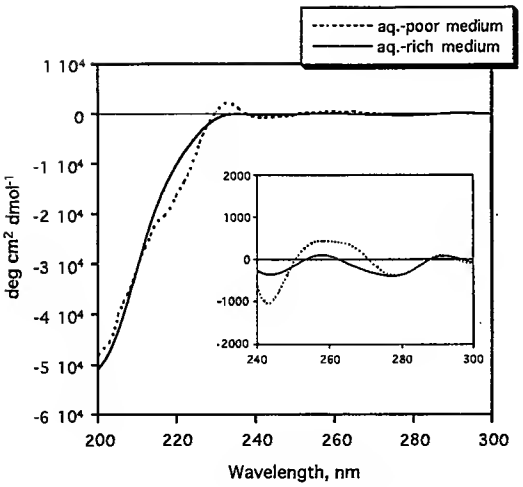

Fig. 9. CD spectra of deamino oxytocin.

band can be assigned to the B_{1u} transition of the tyrosine side chain and the band lying at a shorter wavelength to the n-π^* transition of the amide group (a negative band at 230–240 nm can also be assigned to this transition). Bands at about 250 and 280 nm are due to disulfide group and tyrosine B_{2u} transition, respectively [38–40,51–53].

We surmised that in the aqueous-rich medium, the tested peptides would manifest CD spectra corresponding to less ordered structures than would be found in the aqueous-poor medium. This is especially true in the case of deamino oxytocin. The positive band due to B_{1u} aromatic transition, which is clearly observed in a medium containing only 10% water, completely disappeared when 80% water was used (Fig. 9). The difference in disulfide bands indicates that the flexibility of the disulfide group increases when going from the aqueous-poor to the aqueous-rich medium.

CD spectra of [Asu^{1,6}]-oxytocin also differed markedly, depending on solvent composition (Fig. 10). A moderate positive composite band was seen in the aqueous-rich medium; in the aqueous-poor medium this band clearly dissociates into a strong positive band at about 232 nm and a negative shoulder at about 216 nm. The change of sign of the band corresponding to the amide n-π^* transition was observed by Fric et al. for [Cle^8]-oxytocin and [Pen^1,Cle^8]-oxytocin [53]. This change was supposed to be explained by the competition for the space over the 20-membered ring moiety between the tyrosine side chain and some part of the C-terminal linear tripeptide. Taking into account the much weaker bands of [Asu^{1,6}]-oxytocin compared to that of [Cle^8]-oxytocin and [Pen^1, Cle^8]-oxytocin, we can state that, in our case, we observed only partial restriction of mobility of the tail region of [Asu^{1,6}]-oxytocin.

Thus, the CD measurements gave us some grounds for a proposed explanation of the different chromatographic behaviors of the peptides, depending on the composition of the mobile phase. Probably, in an aqueous-poor medium the C-terminal linear tripeptide of deamino oxytocin and [Asu^{1,6}]-oxytocin interacts quite strongly with the tocin ring; this could occur through the above-mentioned weak hydrogen bond between the Cys^6 CO and the Gly^9 peptide NH, a hydrogen bond between the Asn^5 amide carbonyl and the Leu^8 NH [36], or some other interaction. In an aqueous-rich medium, the acyclic tail of the peptides tends to form conformations with exposed polar groups. In the structure of [Asu^{1,6}]-oxytocin, the presence of the aliphatic portion of aminosuberic acid instead of the disulfide group favors the strengthening of hydrophobic interactions and therefore decreases somewhat the flexibility of the acyclic tail. Therefore, even in the aqueous-rich medium, [Asu^{1,6}]-oxytocin is retained more weakly by the MIP than is oxytocin or deamino oxytocin.

Thus, we showed that it is not only the C-terminal linear tripeptide that can influence the process of molecular recognition of oxytocin and oxytocin analogs by the MIP. Due to different intermolecular (for

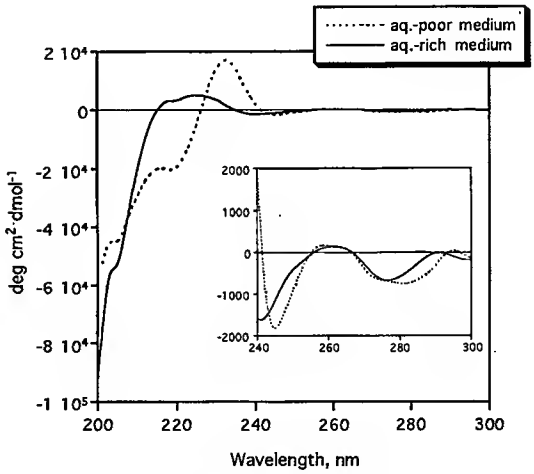

Fig. 10. CD spectra of [Asu^{1,6}]-oxytocin.

example, the case of nonspecific peptides containing the N-terminal amino group in the aqueous-poor mobile phase) or intramolecular interactions (for example, the case of conformational flexibility of deamino oxytocin and [Asu^{1,6}]-oxytocin in the aqueous-rich mobile phase) caused by other parts of the molecules, the final retention pattern seems to be rather complicated. Nevertheless, it is possible to adjust the composition of the chromatographic mobile phase for selective recognition. Moreover, the presence of different 'excess' parts in the structures of the oxytocin and oxytocin analogs allows efficient separation of the different peptides bearing the templated N-terminal sequence.

In conclusion, we have presented here a new approach for the synthesis of MIPs selective to peptides or proteins and have demonstrated the successful application of the proposed method. Using a small tetrapeptide as a template, we synthesized MIPs that were able to recognize oxytocin, a larger peptide possessing the same structural fragment as that of the template. This approach for the development of MIPs selective to proteins is also attractive from an economic viewpoint; a small peptide is usually less expensive, and the quantity necessary for polymer preparation is more readily available than that of the corresponding protein. This investigation is one of the first steps toward the creation of cost-effective, highly selective, efficient synthetic adsorbents and receptors for a wide variety of proteins.

Acknowledgements

A.R. acknowledges a postdoctoral fellowship from NEDO (New Energy and Industrial Technology Development Organization, Japan).

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